RECOMBINANT PROTEIN PRODUCTION IN BOVINE ADENOVIRUS EXPRESSION VECTOR SYSTEM

Technical Field

The present invention relates novel bovine adenovirus (BAV) expression vector systems in which one or both of the early region 1 (E1) and the early region 3 (E3) gene deletions are replaced by a foreign gene and novel recombinant mammalian cell lines stably transformed with BAV E1 sequences, and therefore, expresses E1 gene products, to allow a bovine adenovirus with an E1 gene deletion replaced by a foreign gene to replicate therein. These materials are used in production of recombinant BAV expressing heterologous (antigenic) polypeptides or fragments for the purpose of live recombinant virus or subunit vaccines or for other therapies.

Background of the Invention

The adenoviruses cause enteric or respiratory infection in humans as well as in domestic and laboratory animals.

The bovine adenoviruses (BAVs) comprise at least nine serotypes divided into two subgroups. These subgroups have been characterized based on enzyme-linked immunoassays (ELISA), serologic studies with immunofluorescence assays, virus-neutralization tests, immunoelectron microscopy, by their host specificity and clinical syndromes. Subgroup 1 viruses include BAV 1, 2, 3 and 9 and grow relatively well in established bovine cells compared to subgroup 2 which includes BAV 4, 5, 6, 7 and 8.

BAV3 was first isolated in 1965 and is the best characterized of the BAV genotypes and contains a genome of approximately 35 kb (Kurokawa et al (1978) J. Virol. 28:212-218). The locations of hexon (Hu et al (1984) J. Viol. 49:604-608) and proteinase (Cai et al., (1990) Nuc. Acids Res., 18:5568), genes in the BAV3 genome have been identified and sequenced. However, the location and sequences of other genes such as early region 1 (E1) and 3 (E3) in the BAV genome have not been reported.

In the human adenovirus (HAd) genome there are two important regions: E1 and E3 in which foreign genes can be inserted to generate recombinant adenoviruses (Berkner and Sharp (1984) Nuc. Acid Res., 12:1925-1941 and Haj-Ahmad and Graham (1986) J. Virol., 57:267-274). E1 proteins are essential for virus replication in tissue culture, however, conditional-helper adenovirus recombinants containing foreign DNA in the E1 region, can be generated in a cell line which constitutively expresses E1 (Graham et al., (1977) J. Gen. Virol., 36:59-72). In contrast, E3 gene products of HAd 2 and HAd 5 are not required for in vitro or in vivo infectious virion production, but have an important role in host immune responses to virus infection (Andersson et al (1985) Cell 43:215-222; Burgert et al (1987) EMBO J. 6:2019-2026; Carlin et al (1989) Cell 57:135-144; Ginsberg et al (1989) PNAS, USA 86:3823-3827; Gooding et al (1988) Cell 53:341-346; Tollefson et al (1991) J. Virol. 65:3095-3105; Wold and Gooding (1989) Mol. Biol. Med. 6:433-452 and Wold and Gooding (1991) Virology 184:1-8). The E3-19kiloDalton (kDa) glycoprotein (gp19) of human adenovirus type 2 (HAd2) binds to the heavy chain of a number of class 1 major histocompatibility complex (MHC) antigens in the endoplasmic reticulum thus inhibiting their transport to the plasma membrane (Andersson et al. (1985) Cell 43:215-222; Burgert and

Kvist, (1985) Cell 41:987-997; Burgert and Kvist, (1987) EMBO J. 6:2019-2026). The E3-14.7kDa protein of HAd2 or HAd5 prevents lysis of virus-infected mouse cells by tumor necrosis factor (TNF) (Gooding et al. (1988) Cell 53:341-346). In addition, the E3-10.4kDa and E3-14.5kDa proteins form a complex to induce endosomal-mediated internalization and degradation of the epidermal growth factor receptor (EGF-R) in virus-infected cells (Carlin et al. Cell 57:135-144; Tollefson et al. (1991) J. Virol. 65:3095-3105). The helper-independent recombinant adenoviruses having foreign genes in the E3 region replicate and express very well in every permissive cell line (Chanda et al (1990) Virology 175:535-547; Dewar et al (1989) J. Virol. 63:129-136; Johnson et al (1988) Virology 164:1-14; Lubeck et al (1989) PNAS, USA 86:6763-6767; McDermott et al (1989) Virology 169:244-247; Mittal et al (1993) Virus Res. 28:67-90; Morin et al (1987) PNAS, USA 84:4626-4630; Prevec et al (1990) J. Inf. Dis. 161:27-30; Prevec et al (1989) J. Gen. Virol. 70:429-434; Schneider et al (1989) J. Gen. Virol. 70:417-427 and Yuasa et al (1991) J. Gen. Virol. 72:1927-1934). Based on the above studies and the suggestion that adenoviruses can package approximately 105% of the wild-type (wt) adenovirus genome (Bett et al (1993) J. Virol. 67:5911-5921 and Ghosh-Choudhury et al (1987) EMBO. J. 6:1733-1739), an insertion of up to 1.8 kb foreign DNA can be packaged into adenovirus particles for use as an expression vector for foreign proteins without any compensating deletion.

It is assumed that an indigenous adenovirus vector would be better suited for use as a live recombinant virus vaccine in different animal species compared to an adenovirus of human origin. Non-human adenovirus-based expression vectors have not been reported so far. If like HAds E3, the E3 regions in

other adenoviruses are not essential for virus replication in cultured cells, adenovirus recombinants containing foreign gene inserts in the E3 region could be generated.

BAV3 is a common pathogen of cattle usually resulting in subclinical infection though occasionally associated with a more serious respiratory tract infection (Darbyshire et al., 1966 Res. Vet Sci 7:81-93; Mattson et al., 1988 J. Vet Res 49:67-69). BAV3 can produce tumors when injected into hamsters (Darbyshire, 1966 Nature 211:102) and viral DNA can efficiently effect morphological transformation of mouse, hamster or rat cells in culture (Tsukamoto and Sugino, 1972 J. Virol. 9:465-473; Motoi et al., 1972 Gann 63:415-418; M. Hitt, personal communication). Cross hybridization was observed between BAV3 and human adenovirus type 2 (HAd2) (Hu et al., 1984 J. Virol. 49:604-608) in most regions of the genome including some regions near but not at the left end of the genome.

The E1A gene products of the group C human adenoviruses have been very extensively studied and shown to mediate transactivation of both viral and cellular genes (Berk et al., 1979 Cell 17:935-944; Jones and Shenk, 1979 Cell 16:683-689; Nevins, 1981 Cell 26:213-220; Nevins, 1982 Cell 29:913-919; reviewed in Berk, 1986 Ann. Res. Genet 20:45-79), to effect transformation of cells in culture (reviewed in Graham, F.L. (1984) "Transformation by and oncogenicity of human adenoviruses. In:The Adenoviruses." H.S. Ginsberg, Editor. Plenum Press, New York; Branton et al., 1985 Biochim. Biophys. Acta 780:67-94) and induce cell DNA synthesis and mitosis (Zerler et al., 1987 Mol. Cell Biol. 7:821-929; Bellet et al., 1989 J. Virol. 63:303-310; Howe et al., 1990 PNAS, USA 87:5883-5887; Howe and Bayley, 1992 Virology 186:15-24). The E1A transcription

unit comprises two coding sequences separated by an intron region which is deleted from all processed E1A transcripts. In the two largest mRNA species produced from the E1A transcription unit, the first coding regions is further subdivided into exon 1, a sequence found in both the 12s and 13s mRNA species, and the unique region, which is found only in the 13s mRNA species. By comparisons between E1A proteins of human and simian adenoviruses three regions of somewhat conserved protein sequence (CR) have been defined (Kimelman et al., 1985 J. Virol. 53:399-409). CR1 and CR2 are encoded in exon 1, while CR3 is encoded in the unique sequence and a small portion of exon 2. Binding sites for a number of cellular proteins including the retinoblastoma protein Rb, cyclin A and an associated protein kinase $p33^{cdk2}$, and other, as yet unassigned, proteins have been defined in exon 1 encoded regions of E1A proteins (Yee and Branton, 1985 Virology 147:142-153; Harlow et al., 1986 Mol. Cell Biol. 6:1579-1589; Barbeau et al., 1992 Biochem. Cell Biol. 70:1123-1134). Interaction of E1A with these cellular proteins has been implicated as the mechanism through which E1A participates in immortalization and oncogenic transformation (Egan et al, 1989 Oncogene 4:383-388; Whyte et al., 1988 Nature 334:124-129; Whyte et al, 1988 J. Virol. 62:257-265). While E1A alone may transform or immortalize cells in culture, the coexpression of both E1A and either the E1B-19k protein or the E1B-55k protein separately or together is usually required for high frequency transformation of rodent cells in culture (reviewed in Graham, 1984 supra; Branton et al., 1985 supra; McLorie et al., 1991 J. Gen Virol. 72:1467-1471).

Transactivation of other viral early genes in permissive infection of human cells is principally mediated by the amino acid sequence encoded in the CR3

region of E1A (Lillie et al., 1986 Cell 46:1043-1051). Conserved cysteine residues in a $CysX_2CysX_{13}CysX_2Cys$ sequence motif in the unique region are associated with metal ion binding activity (Berg, 1986 supra) and are essential for transactivation activity (Jelsma et al., 1988 Virology 163:494-502; Culp et al., 1988 PNAS, USA 85:6450-6454). As well, the amino acids in CR3 which are immediately amino (N)-terminal to the metal binding domain have been shown to be important in transcription activation, while those immediately carboxy (C)-terminal to the metal binding domain are important in forming associations with the promoter region (Lillie and Green, 1989 Nature 338:39-44; see Fig. 3).

The application of genetic engineering has resulted in several attempts to prepare adenovirus expression systems for obtaining vaccines. Examples of such research include the disclosures in U.S. patent 4,510,245 on an adenovirus major late promoter for expression in a yeast host; U.S. patent 4,920,209 on a live recombinant adenovirus type 7 with a gene coding for hepatitis-B surface antigen located at a deleted early region 3; European patent 389 286 on a non-defective human adenovirus 5 recombinant expression system in human cells for HCMV major envelope glycoprotein; WO 91/11525 on live non-pathogenic immunogenic viable canine adenovirus in a cell expressing E1a proteins; French patent 2 642 767 on vectors containing a leader and/or promoter from the E3 of adenovirus 2.

The selection of a suitable virus to act as a vector for foreign gene expression, and the identification of a suitable non-essential region as a site for insertion of the gene pose a challenge. In particular, the insertion site must be non-essential for the viable replication of the virus and its effective operation in tissue culture and also in vivo. Moreover,

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the insertion site must be capable of accepting new genetic material, whilst ensuring that the virus continues to replicate. An essential region of a virus genome can also be utilized for foreign gene insertion if the recombinant virus is grown in a cell line which complements the function of that particular essential region in trans.

The present inventors have now identified suitable regions in the BAV genome and have succeeded in inserting foreign genes to generate BAV recombinants.

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Disclosure of the Invention

The present invention relates to novel bovine adenovirus expression vector systems in which part or all of one or both of the E1 and E3 gene regions are deleted and to recombinant mammalian cell lines of bovine origin transformed with the BAV E1 sequences, and thus, constitutively express the E1 gene products to allow bovine adenovirus, having a deletion of part or all of the E1 gene region replaced by a heterologous nucleotide sequence encoding a foreign gene or fragment thereof, to replicate therein and use of these materials in production of heterologous (antigenic) polypeptides or fragments thereof.

The invention also related to a method of preparing a live recombinant virus or subunit vaccines for producing antibodies or cell mediated immunity to an infectious organism in a mammal, such as bovine, which comprises inserting into the bovine adenovirus genome the gene or fragment coding for the antigen which corresponds to said antibodies or induces said cell mediated immunity, together with or without an effective promoter therefore, to produce BAV recombinants.

Generally, the foreign gene construct is cloned into a nucleotide sequence which represents only a part

of the entire viral genome having one or more appropriate deletions. This chimeric DNA sequence is usually present in a plasmid which allows successful cloning to produce many copies of the sequence. The cloned foreign gene construct can then be included in the complete viral genome, for example, by in vivo recombination following a DNA-mediated cotransfection technique. Multiple copies of a coding sequence or more than one coding sequences can be inserted so that the recombinant vector can express more than one foreign protein. The foreign gene can have additions, deletions or substitutions to enhance expression and/or immunological effects of the expressed protein.

The invention also includes an expression system comprising an bovine adenovirus expression vector wherein heterologous nucleotide sequences with or without any exogenous regulatory elements, replace the E1 gene region and/or part or all of the E3 gene region.

The invention also includes (A) a recombinant vector system comprising the entire BAV DNA and a plasmid or two plasmids capable of generating a recombinant virus by in vivo recombination following cotransfection of a suitable cell line comprising BAV DNA representing the entire wild-type BAV genome and a plasmid comprising a bovine adenovirus left or right end sequences containing the E1 or E3 gene regions, respectively, with a heterologous nucleotide sequence encoding a foreign gene or fragment thereof substituted for part or all of the E1 or E3 gene regions; (B) a live recombinant bovine adenovirus vector (BAV) system selected from the group consisting of: (a) a system wherein part or all of the E1 gene region is replaced by a heterologous nucleotide sequence encoding a foreign gene or fragment thereof; (b) a system wherein a part or all of the E3 gene region is replaced by a heterologous nucleotide sequence encoding a

foreign gene or fragment thereof; and (c) a system wherein part or all of the E1 gene region and part or all of the E3 gene region are deleted and a heterologous nucleotide sequence encoding a foreign gene or fragment thereof is inserted into at least one of the deletions; (C) a recombinant bovine adenovirus (BAV) comprising a deletion of part or all of E1 gene region, a deletion of part or all of E3 gene region or deletion of both, and inserted into at least one deletion a heterologous nucleotide sequence coding for an antigenic determinant of a disease causing organism; (D) a recombinant bovine adenovirus expression system comprising a deletion of part or all of E1, a deletion of part or all of E3, or both deletions, and inserted into at least one deletion a heterologous nucleotide sequence coding for a foreign gene or fragment thereof under control of an expression promoter: or (E) a recombinant bovine adenovirus (BAV) for producing an immune response in a mammalian host comprising: (1) BAV recombinant containing a heterologous nucleotide sequence coding for an antigenic determinant needed to obtain the desired immune response in association with or without (2) an effective promoter to provide expression of said antigenic determinant in immunogenic quantities for use as a live recombinant virus or recombinant protein or subunit vaccine; (F) a mutant bovine adenovirus (BAV) comprising a deletion of part or all of E1 and/or a deletion of part or all of E3.

Recombinant mammalian cell lines stably transformed with BAV E1 gene region sequences, said recombinant cell lines thereby capable of allowing replication therein of a bovine adenovirus comprising a deletion of part or all of the E1 or E3 gene regions replaced by a heterologous or homologous nucleotide sequence encoding a foreign gene or fragment thereof. The invention also includes production, isolation and

purification of polypeptides or fragments thereof, such as growth factors, receptors and other cellular proteins from recombinant bovine cell lines expressing BAV E1 gene products.

The invention also includes a method for providing gene therapy to a mammal in need thereof to control a gene deficiency which comprises administering to said mammal a live recombinant bovine adenovirus containing a foreign nucleotide sequence encoding a non-defective form of said gene under conditions wherein the recombinant virus vector genome is incorporated into said mammalian genome or is maintained independently and extrachromosomally to provide expression of the required gene in a target organ or tissue.

Another aspect of the invention provides a virus vaccine composition which comprises the recombinant virus or recombinant protein in association with or without a pharmaceutically acceptable carrier. The recombinant virus vaccine can be formulated for administration by an oral dosage (e.g. as an enteric coated tablet), by injection or otherwise. More specifically, these include a vaccine for protecting a mammalian host against infection comprising a live recombinant adenovirus or recombinant protein produced by the recombinant adenovirus of the invention wherein the foreign gene or fragment encodes an antigen and formulated with or without a pharmaceutically acceptable carrier.

The invention also includes methods of producing antibodies or cell mediated immunity in a mammal including (1) a method for eliciting an immune response in a mammalian host against an infection comprising: administering a vaccine comprising a live BAV recombinant of the invention wherein the foreign gene or

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fragment encodes an antigen with or without a pharmaceutically acceptable carrier, and (2) a method for eliciting an immune response in a mammalian host against an infection comprising: administering a vaccine comprising a recombinant antigen prepared by culturing a BAV recombinant wherein the foreign gene or fragment encodes the desired antigen with or without a pharmaceutically acceptable carrier.

The following disclosure will render these and other embodiments of the present invention readily apparent to those of skill in the art. While the disclosure often refers to bovine adenovirus type 3 (BAV3), it should be understood that this is for the purpose of illustration and that the same features apply to bovine adenovirus of the other type, 1, 2, 4, 5, 6, 7 8, and 9 and the invention described and claimed herein is intended to cover all of these bovine adenovirus types.

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Brief Description of the Drawings

Figure 1. Sequence and major open reading frames of the left 11% of the BAV3 genome. The region comprises the E1 and protein IX transcription region. The 195 nucleotide inverted terminal repeat sequence identified by Shinagawa et al., 1987 Gene 55:85-93 is shown in *italics*. The amino acid sequence for the largest E1A protein, two E1B proteins and protein IX are presented. The probable splice donor ([), splice acceptor (]) and intron sequence (*underlined italics*) within the E1A region are marked. A 35 base pair repeat sequence between E1A and E1B is indicated in **bold underline**. Possible transcription promoter **TATA** sequences and possible poly A addition sequences **AATAA** are also indicated.

Figure 2. Regions of homology in the E1A proteins of BAV3 and human adenovirus type 5 (HAd5). The amino acid residue of each serotype is indicated. A. Conserved region 3 (CR3) of HAd5 subdivided into three functional regions as defined by Lillie et al (1989) Nature 338:39-44 and described in the Background of the Invention. The intron sequence of BAV3 E1A occurs within the serine amino acid codon at position 204. B. A portion of conserved region 2 (CR2) of HAd5, showing the residues thought to be important in the binding of retinoblastoma protein Rb (Dyson et al., 1990 J. Virol. 64:1353-1356), and the comparable sequence from BAV3.

Figure 3. Homology regions between the HAd5 and E1B 19k (176R) protein and the corresponding BAV3 (157R) protein. The amino acid residue number for each of the viruses is indicated.

Figure 4. The C-terminal 346R of HAd5 E1B 56k (496R) and the corresponding BAV3 protein (420R). The HAd5 protein comparison begins at residue 150 and the BAV3 (in italics) at residue 74. The amino terminal regions of these proteins which are not presented show no significant homology.

Figure 5. Homology comparison of the amino acid sequence of HAd5 protein IX and the corresponding protein of BAV3 (in italics).

Figure 6. The genome of BAV3 showing the location of *Eco*RI, *Xba*I and *BAM*HI sites and the structure of the 5100bp segment from 77 to 92 m.u. ORFs for the upper strand which can encode 60 amino acids or more are represented by bars. Shaded portions indicate regions of similarity to pVIII, 14.7K E3 and fibre proteins of HAd2 or -5. The first methionine followed by a stretch of amino acids of at least 50 is shown by an open triangle. Termination codons for ORFs likely to code for viral proteins are shown by closed triangles.

Figure 7. Nucleotide sequence of BAV3 between 77 and 92 m.u. showing ORFs that have the potential to encode polypeptides of at least 50 amino acids after the initiating methionine. The nucleotide sequence was analyzed using the program DISPCOD (PC/GENE). Potential N-glycosylation sites (N-X-T/S) and polyadenylation signals are underlined and the first methionine of each ORF is shown in bold.

Figure 8. Comparison between the predicted amino acid sequences for the ORFs of BAV3 and known proteins of HAd2 or -5 using the computer program PALIGN (PC/GENE), with comparison matrix structural-genetic matrix; open gap cost 6; unit gap cost 2. Identical residues are indicated by a colon and similar residues by a dot. (a) Comparison between the predicted amino acid sequence encoded by the 3′ end of BAV3 ORF 1 and the HAd2 hexon-associated pVIII precursor. (b) Comparison between the ORF 4 and the HAd5 14.7K E3 protein. (c) Comparison between the predicted amino acid sequence encoded by BAV3 ORF 6 and the HAd2 fibre protein.

Figure 9. Construction of BAV3 E3 transfer vector containing the firefly luciferase gene. The 3.0 kb BamHI 'D' fragment of the BAV3 genome which falls between m.u. 77.8 and 86.4, contains almost the entire E3 region (Mittal et al (1992) J. Gen. Virol. 73:3295-3000). This 3.0 kb fragment was isolated by digesting BAV3 DNA with BamHI and cloned into pUC18 at the BamHI site to obtain pSM14. Similarly, the 4.8 kb BamHI 'C' fragment of BAV3 DNA which extends between m.u. 86.4 and 100 was isolated and inserted into pUC18 to produce pSM17. To delete a 696 bp XhoI-NcoI fragment, pSM14 was cleaved with XhoI and NcoI, the larger fragment was purified and the ends were made blunt with Klenow fragment of DNA polymerase I and a NruI-SalI linker was inserted to generate pSM14de12. A 2.3 kb BamHI fragment containing

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BAV3 sequences, an E3 deletion and NruI and SalI cloning sites, was inserted into pSM17 at the BamHI site to obtain pSM41, however, this step was not required for construction of a BAV3 E3 transfer vector. A 1716 bp fragment containing the firefly luciferase gene (de Wet et al (1987) Mol. Cell. Biol. 7:725-737) was isolated by digesting pSVOA/L (provided by D. R. Helinski, University of California at San Diego, CA) with BsmI and SspI as described (Mittal et al (1993) Virus Res. 28:67-90), and the ends were made blunt with Klenow. The luciferase gene was inserted into pSM41 at the SalI site by blunt end ligation. The resultant plasmid was named pSM41-Luc which contained the luciferase gene in the same orientation as the E3 transcription unit. The plasmid pKN30 was digested with XbaI and inserted into pSM41-Luc (partially cleaved with XbaI) at a XbaI site present within the luciferase gene to obtain pSM41-Luc-Kan. The plasmid pSM14 was digested with BamHI and a 3.0 kb fragment was isolated and inserted into pSM17 at the BamHI site to generate pSM43. The 18.5 kb XbaI 'A' fragment of the BAV3 genome which falls between m.u. 31.5 and 84.3 was cloned into pUC18 at the XbaI site to result pSM21. A 18.5 kb XbaI fragment was purified from pSM21 after cleavage with XbaI and inserted into pSM43 at the XbaI site and the resultant plasmid was named pSM51. A 7.7 kb BamHI fragment containing the luciferase gene and kan^r gene was isolated after digesting pSM41-Luc-Kan with BamHI and ligated to pSM51, partially digested with BamHI, to isolate pSM51-Luc-Kan in the presence of ampicillin and kanamycin. Finally the kan^r gene was deleted from pSM51-Luc-Kan by partial cleavage with XbaI and religation to obtain pSM51-Luc.

Figure 10. Generation of BAV3 recombinants containing the firefly luciferase in the E3 region. The plasmid pSM51-Luc contains the BAV3 genome between m.u.

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77.8-84.3 and 31.5-100, a 696 bp deletion in E3 and the luciferase gene in E3 in the E3 parallel orientation. The BAV3 genome digested with PvuI and uncut pSM51-Luc were used for cotransfection of MDBK cells transformed with a plasmid containing BAV3 E1 sequences to rescue the luciferase gene in E3 of the BAV3 genome by *in vivo* recombination. The resulting BAV3-luciferase recombinants (BAV3-Luc) isolated from two independent experiments were named BAV3-Luc (3.1) and BAV3-Luc (3.2). The BamHI restriction map of the BAV3-Luc genome is shown. The position and orientation of the firefly luciferase gene is shown as a hatched arrow.

Figure 11. Southern blot analyses of restriction enzymes digested DNA fragments of the wt BAV3 or recombinant genomes by using a 696 bp XhoI-NcoI fragment from pSM14 (Fig. 9) and a DNA fragment containing the luciferase gene as probes. 100 ng DNA isolated from the mock (lanes 1, 2, 3), BAV3-Luc (3.1) (lanes 4, 5, 6), BAV3-Luc (3.2) (lanes 7, 8, 9) or wt BAV3 (lanes 10, 11 12)-infected MDBK cells were digested with BamHI (lanes 1, 4, 7, 10), EcoRI (lanes 2, 5, 8, 11) or XbaI (lanes 3, 6, 9, 12) and analyzed by agarose gel electrophoresis. The DNA fragments from the gel were transferred onto a *GeneScreenPlus*™ membrane and hybridized with a 696 bp XhoI-NcoI fragment from pSM14 (Fig. 9) labeled with ^{32}P using Pharmacia Oligolabeling Kit (panel A). Panel B blot represents duplicate samples as in panel A but was probed with a 1716 bp BsmI-SspI fragment containing the luciferase gene (Fig. 9). The sizes of bands visualized following hybridization are shown in kb on the right in panel A and on the left in panel B.

B: BamHI, E: EcoRI, Xb: XbaI, 3.1: BAV3-Luc (3.1), 3.2: BAV3-Luc (3.2) and wt: wild-type BAV3.

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Figure 12. Single step growth curve for wt BAV3 and BAV3-Luc. Confluent monolayers of MDBK cells in 25 mm multi-well culture plates were inoculated with the wt BAV3, BAV3-Luc (3.1) or BAV3-Luc (3.2) at a m.o.i. of 10 p.f.u. per cell. The virus was allowed to adsorb for 1 h at 37°C, cell monolayers were washed 3 times with PBS^{++} (0.137 M NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, containing 0.01% $CaCl_2.2H_2O$ & 0.01% $MgCl_2.6H_2O$) and incubated at 37°C in 1 ml maintenance medium containing 2% horse serum. At various times post-infection, cells were harvested along with the supernatant, frozen and thawed three times and titrated on MDBK cells by plaque assay. Results are the means of duplicate samples.

Figure 13. Kinetics of luciferase expression in MDBK cells-infected with BAV3-Luc. Confluent MDBK cell monolayers in 25 mm multi-well culture plates were infected with BAV3-Luc (3.1) or BAV3-Luc (3.2) at a m.o.i. of 50 p.f.u. per cell. At indicated time points post-infection, virus-infected cells were harvested and assayed in duplicate for luciferase activity.

Figure 14. Luciferase expression in the presence of 1-β-D-arabinofluranosyl cytosine (AraC) in MDBK cells-infected with BAV3-Luc. Confluent MDBK cell monolayers in 25 mm multi-well culture plates were infected with A) BAV3-Luc (3.1) or B) BAV3-Luc (3.2) at a m.o.i. of 50 p.f.u. per cell and incubated in the absence or presence of 50 μg AraC per ml of maintenance medium. At indicated time points post-infection, virus-infected cells were harvested and assayed in duplicate for luciferase activity.

Figure 15. Transcription maps of the wt BAV3 and BAV3-Luc genomes in the E3 region. The genome of wt BAV3 between m.u. 77 and 82 is shown which represents the E3 region. The location of XhoI and NcoI sites which were used to make an E3 deletion are shown. (a) The

three frames (F1, F2 and F3) representing the open reading frames (ORFs) in the upper strand of the wt BAV3 genome in the E3 region are represented by bars. The shaded portions indicate regions of similarities to pVIII and E3-14.7 kDa proteins of HAd5. The positions of the initiation and termination codons for ORFs likely to code for viral proteins are shown by open and closed triangles, respectively. (b) The predicted ORFs for the upper strand in E3 of the BAV3-Luc genome are shown after a 696 bp XhoI-NcoI E3 deletion replaced by the luciferase gene. The ORFs for pVIII and E3-14.7 kDa proteins are intact. The transcription map of the wt BAV3 E3 was adapted from the DNA sequence submitted to the GenBank database under accession number D16839.

Figure 16. Western blot analysis of virus-infected MDBK cells using an anti-luciferase antibody. Confluent monolayers of MDBK cells were mock-infected (lane 1) or infected with the wt BAV3 (lane 2), BAV3-Luc (3.1) (lane 3) and BAV3-Luc (3.2) (lane 4) at a m.o.i. of 50 p.f.u. per cell, harvested at 18 h post-infection, cell extracts prepared and analyzed by SDS-PAGE and Western blotting using a rabbit anti-luciferase antibody. Purified firefly luciferase was used as a positive control (lane 5). The lane 5 was excised to obtain a shorter exposure. The protein molecular weight markers in kDa are shown on the left. The arrow indicates the 62 kDa luciferase bands reacted with the anti-luciferase antibody.
wt: wild-type BAV3, 3.1: BAV3-Luc (3.1) and 3.2: BAV3-Luc (3.2).

Figure 17. Construction of pSM71-neo. A 8.4 kb SalI fragment of the BAV3 genome which falls between m.u. 0 and 24 was isolated and inserted into pUC19 at the SalI-SmaI site to generate pSM71. The plasmid, pRSDneo (Fitzpatrick et al (1990) Virology 176:145-157) contains

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the neomycin-resistant (neo^r gene flanked with the simian virus 40 (SV40) regulatory sequences originally from the plasmid, pSV2neo (Southern et al (1982) J. Mol. Appl. Genet 1:327-341) after deleting a portion of the SV40 sequences upstream of the neo^r gene to remove several false initiation codons. A 2.6 kb fragment containing the neo^r gene under the control of the SV40 regulatory sequences, was obtained from the plasmid, pRSDneo after digestion with BamHI and Bg1II, and cloned into pSM71 at the SalI site by blunt end ligation to obtain pSM71-neo containing the neo^r gene in the E1 parallel orientation.

Figure 18. Construction of pSM61-kan 1 and pSM61-kan2. A 11.9 kb Bg1II fragment of the BAV3 genome which extends between m.u. 0 and 34 was purified and introduced into pUC19 at the BamHI-HincII site to obtain pSM61. The plasmid, pKN30 contains the neo^r gene along with SV40 promoter and polyadenylation sequences from the plasmid pSV2neo without any modification. The entire pKN30 plasmid was inserted into pSM61 at the SalI site to generate pSM61-kan1 having the neo^r gene in the E1 anti-parallel orientation and pSM61-kan2 when the neo^r gene is in the E1 parallel orientation.

Figure 19. Construction of an E1 transfer plasmid containing the beta-galactosidase gene.

The plasmid, pSM71 which contains the BAV3 genome between m.u. 0 and 24, was cleaved with ClaI and partially with AvrII to delete a 2.6 kb AvrII-ClaI fragment (between m.u. 1.3 and 8.7) which falls within the E1 region. A 0.5 kb fragment containing the SV40 promoter and polyadenylation sequences was obtained from pFG144K5-SV by digesting with XbaI and inserted into pSM71 to replace the 2.6 kb deletion to generate pSM71-del1-SV. A 3.26 kb fragment containing the bacterial beta-galactosidase gene was isolated from pDUC/Z (Liang et al (1993) Virology 195:42-50) after cleavage with NcoI

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and HindIII and cloned into pSM71-del1-SV at the BamHI site to put the beta-galactosidase gene under the control of the SV40 regulatory sequences to obtain pSM71-Z.

Modes of Carrying Out the Invention

The practice of the present invention will employ, unless otherwise indicated, conventional microbiology, immunology, virology, molecular biology, and recombinant DNA techniques which are within the skill of the art. These techniques are fully explained in the literature. See, e.g., Maniatis et al., Molecular Cloning: A Laboratory Manual (1982); DNA Cloning: A Practical Approach, vols. I & II (D. Glover, ed.); Oligonucleotide Synthesis (N. Gait, ed. (1984)); Nucleic Acid Hybridization (B. Hames & S. Higgins, eds. (1985)); Transcription and Translation (B. Hames & S. Higgins, eds. (1984)); Animal Cell Culture (R. Freshney, ed. (1986)); Perbal, A Practical Guide to Molecular Cloning (1984). Sambrook et al., Molecular Cloning: A Laboratory Manual (2nd Edition); vols. I, II & III (1989).

A. Definitions

In describing the present invention, the following terminology, as defined below, will be used.

A "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication in vivo; i.e., is capable of replication under its own control.

A "vector" is a replicon, such as a plasmid, phage, cosmid or virus, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

By "live virus" is meant, in contradistinction to "killed" virus, a virus which is capable of producing

identical progeny in tissue culture and inoculated animals.

A "helper-free virus vector" is a vector that does not require a second virus or a cell line to supply something defective in the vector.

A "double-stranded DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in its normal, double-stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, inter alia, in linear DNA molecules (e.g., restriction fragments of DNA from viruses, plasmids, and chromosomes). In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5′ to 3′ direction along the nontranscribed strand of DNA (i.e., the strand having the sequence homologous to the mRNA).

A DNA "coding sequence" is a DNA sequence which is transcribed and translated into a polypeptide <u>in vivo</u> when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5′ (amino) terminus and a translation stop codon at the 3′ (carboxy) terminus. A coding sequence can include, but is not limited to, procaryotic sequences, cDNA from eucaryotic mRNA, genomic DNA sequences from eucaryotic (e.g., mammalian) DNA, viral DNA, and even synthetic DNA sequences. A polyadenylation signal and transcription termination sequence will usually be located 3′ to the coding sequence.

A "transcriptional promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3′

direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bound at the 3′ terminus by the translation start codon (ATG) of a coding sequence and extends upstream (5′ direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eucaryotic promoters will often, but not always, contain "TATA" boxes and "CAAT" boxes. Procaryotic promoters contain Shine-Dalgarno sequences in addition to the -10 and -35 consensus sequences.

DNA "control sequences" refer collectively to promoter sequences, ribosome binding sites, polyadenylation signals, transcription termination sequences, upstream regulatory domains, enhancers, and the like, which collectively provide for the transcription and translation of a coding sequence in a host cell.

A coding sequence or sequence encoding is "operably linked to" or "under the control of" control sequences in a cell when RNA polymerase will bind the promoter sequence and transcribe the coding sequence into mRNA, which is then translated into the polypeptide encoded by the coding sequence.

A "host cell" is a cell which has been transformed, or is capable of transformation, by an exogenous DNA sequence.

A cell has been "transformed" by exogenous DNA when such exogenous DNA has been introduced inside the cell membrane. Exogenous DNA may or may not be integrated (covalently linked) to chromosomal DNA making up the genome of the cell. In procaryotes and yeasts,

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for example, the exogenous DNA may be maintained on an episomal element, such as a plasmid. A stably transformed cell is one in which the exogenous DNA has become integrated into the chromosome so that it is inherited by daughter cells through chromosome replication. For mammalian cells, this stability is demonstrated by the ability of the cell to establish cell lines or clones comprised of a population of daughter cell containing the exogenous DNA.

A "clone" is a population of daughter cells derived from a single cell or common ancestor. A "cell line" is a clone of a primary cell that is capable of stable growth _in vitro_ for many generations.

Two polypeptide sequences are "substantially homologous" when at least about 80% (preferably at least about 90%, and most preferably at least about 95%) of the amino acids match over a defined length of the molecule.

Two DNA sequences are "substantially homologous" when they are identical to or not differing in more that 40% of the nucleotides, more preferably about 20% of the nucleotides, and most preferably about 10% of the nucleotides.

DNA sequences that are substantially homologous can be identified in a Southern hybridization experiment under, for example, stringent conditions, as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. _See, e.g._, Maniatis et al., _supra_; _DNA Cloning_, vols. I & II, _supra_; _Nucleic Acid Hybridization_, _supra_.

A "heterologous" region of a DNA construct is an identifiable segment of DNA within or attached to another DNA molecule that is not found in association with the other molecule in nature. Thus, when the heterologous region encodes a viral gene, the gene will usually be flanked by DNA that does not flank the viral

gene in the genome of the source virus or virus-infected cells. Another example of the heterologous coding sequence is a construct where the coding sequence itself is not found in nature (e.g., synthetic sequences having codons different from the native gene). Allelic variation or naturally occurring mutational events do not give rise to a heterologous region of DNA, as used herein.

"Bovine host" refers to cattle of any breed, adult or infant.

The term "protein" is used herein to designate a polypeptide or glycosylated polypeptide, respectively, unless otherwise noted. The term "polypeptide" is used in its broadest sense, i.e., any polymer of amino acids (dipeptide or greater) linked through peptide bonds. Thus, the term "polypeptide" includes proteins, oligopeptides, protein fragments, analogs, muteins, fusion proteins and the like.

"Fusion protein" is usually defined as the expression product of a gene comprising a first region encoding a leader sequence or a stabilizing polypeptide, and a second region encoding a heterologous protein. It involves a polypeptide comprising an antigenic protein fragment or a full length BAV protein sequence as well as (a) heterologous sequence(s), typically a leader sequence functional for secretion in a recombinant host for intracellularly expressed polypeptide, or an N-terminal sequence that protects the protein from host cell proteases, such as SOD. An antigenic protein fragment is usually about 5-7 amino acids in length.

"Native" proteins or polypeptides refer to proteins or polypeptides recovered from BAV or BAV-infected cells. Thus, the term "native BAV polypeptide" would include naturally occurring BAV proteins and fragments thereof. "Non-native" polypeptides refer to

polypeptides that have been produced by recombinant DNA methods or by direct synthesis. "Recombinant" polypeptides refers to polypeptides produced by recombinant DNA techniques; i.e., produced from cells transformed by an exogenous DNA construct encoding the desired polypeptide.

A "substantially pure" protein will be free of other proteins, preferably at least 10% homogeneous, more preferably 60% homogeneous, and most preferably 95% homogeneous.

An "antigen" refers to a molecule containing one or more epitopes that will stimulate a host's immune system to make a humoral and/or cellular antigen-specific response. The term is also used interchangeably with "immunogen."

A "hapten" is a molecule containing one or more epitopes that does not stimulate a host's immune system to make a humoral or cellular response unless linked to a carrier.

The term "epitope" refers to the site on an antigen or hapten to which a specific antibody molecule binds or is recognized by T cells. The term is also used interchangeably with "antigenic determinant" or "antigenic determinant site."

An "immunological response" to a composition or vaccine is the development in the host of a cellular and/or antibody-mediated immune response to the composition or vaccine of interest. Usually, such a response consists of the subject producing antibodies, B cells, helper T cells, suppressor T cells, and/or cytotoxic T cells directed specifically to an antigen or antigens included in the composition or vaccine of interest.

The terms "immunogenic polypeptide" and "immunogenic amino acid sequence" refer to a polypeptide or amino acid sequence, respectively, which elicit

antibodies that neutralize viral infectivity, and/or mediate antibody-complement or antibody dependent cell cytotoxicity to provide protection of an immunized host. An "immunogenic polypeptide" as used herein, includes the full length (or near full length) sequence of the desired protein or an immunogenic fragment thereof.

By "immunogenic fragment" is meant a fragment of a polypeptide which includes one or more epitopes and thus elicits antibodies that neutralize viral infectivity, and/or mediates antibody-complement or antibody dependent cell cytotoxicity to provide protection of an immunized host. Such fragments will usually be at least about 5 amino acids in length, and preferably at least about 10 to 15 amino acids in length. There is no critical upper limit to the length of the fragment, which could comprise nearly the full length of the protein sequence, or even a fusion protein comprising fragments of two or more of the antigens. The term "treatment" as used herein refers to treatment of a mammal, such as bovine or the like, either (i) the prevention of infection or reinfection (prophylaxis), or (ii) the reduction or elimination of symptoms of an infection. The vaccine comprises the recombinant BAV itself or recombinant antigen produced by recombinant BAV.

By "infectious" is meant having the capacity to deliver the viral genome into cells.

B. General Method

The present invention identifies and provides a means of deleting part or all of the nucleotide sequence of bovine adenovirus E1 and/or E3 gene regions to provide sites into which heterologous or homologous nucleotide sequences encoding foreign genes or fragments thereof can be inserted to generate bovine adenovirus recombinants.

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By "deleting part of" the nucleotide sequence is meant using conventional genetic engineering techniques for deleting the nucleotide sequence of part of the E1 and/or E3 region.

Various foreign genes or coding sequences (prokaryotic, and eukaryotic) can be inserted in the bovine adenovirus nucleotide sequence, e.g.,DNA, in accordance with the present invention, particularly to provide protection against a wide range of diseases and many such genes are already known in the art. The problem heretofore having been to provide a safe, convenient and effective vaccine vector for the genes or coding sequences.

It is also possible that only fragments of nucleotide sequences of genes can be used (where these are sufficient to generate a protective immune response) rather than the complete sequence as found in the wild-type organism. Where available, synthetic genes or fragments thereof can also be used. However, the present invention can be used with a wide variety of genes, fragment and the like, and is not limited to those set out above.

In some cases the gene for a particular antigen can contain a large number of introns or can be from an RNA virus, in these cases a complementary DNA copy (cDNA) can be used.

In order for successful expression of the gene to occur, it can be inserted into an expression vector together with a suitable promoter including enhancer elements and polyadenylation sequences. A number of eucaryotic promoter and polyadenylation sequences which provide successful expression of foreign genes in mammalian cells and how to construct expression cassettes, are known in the art, for example in U.S. patent 5,151,267, the disclosures of which are

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incorporated herein by reference. The promoter is selected to give optimal expression of immunogenic protein which in turn satisfactorily leads to humoral, cell mediated and mucosal immune responses according to known criteria.

The foreign protein produced by expression *in vivo* in a recombinant virus-infected cell may be itself immunogenic. More than one foreign gene can be inserted into the viral genome to obtain successful production of more than one effective protein.

Thus with the recombinant virus of the present invention, it is possible to provide protection against a wide variety of diseases affecting cattle. Any of the recombinant antigenic determinant or recombinant live virus of the invention can be formulated and used in substantially the same manner as described for the antigenic determinant vaccines or an live vaccine vectors.

The antigens used in the present invention can be either native or recombinant antigenic polypeptides or fragments. They can be partial sequences, full-length sequences, or even fusions (e.g., having appropriate leader sequences for the recombinant host, or with an additional antigen sequence for another pathogen). The preferred antigenic polypeptide to be expressed by the virus systems of the present invention contain full-length (or near full-length) sequences encoding antigens. Alternatively, shorter sequences that are antigenic (i.e., encode one or more epitopes) can be used. The shorter sequence can encode a "neutralizing epitope," which is defined as an epitope capable of eliciting antibodies that neutralize virus infectivity in an in vitro assay. Preferably the peptide should encode a "protective epitope" that is capable of raising in the host an "protective immune response;" i.e., an antibody-

and/or a cell-mediated immune response that protects an immunized host from infection.

The antigens used in the present invention, particularly when comprised of short oligopeptides, can be conjugated to a vaccine carrier. Vaccine carriers are well known in the art: for example, bovine serum albumin (BSA), human serum albumin (HSA) and keyhole limpet hemocyanin (KLH). A preferred carrier protein, rotavirus VP6, is disclosed in EPO Pub. No. 0259149, the disclosure of which is incorporated by reference herein.

Genes for desired antigens or coding sequences thereof which can be inserted include those of organisms which cause disease in mammals, particularly bovine pathogens such as bovine rotavirus, bovine coronavirus, bovine herpes virus type 1, bovine respiratory syncytial virus, bovine parainfluenza virus type 3 (BPI-3), bovine diarrhea virus, Pasteurella haemolytica, Haemophilus somnus and the like. The vaccines of the invention carrying foreign genes or fragments can also be orally administered in a suitable oral carrier, such as in an enteric-coated dosage form. Oral formulations include such normally-employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin cellulose, magnesium carbonate, and the like. Oral vaccine compositions may be taken in the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations, or powders, containing from about 10% to about 95% of the active ingredient, preferably about 25% to about 70%. An oral vaccine may be preferable to raise mucosal immunity in combination with systemic immunity, which plays an important role in protection against pathogens infecting the gastrointestinal tract.

In addition, the vaccine be formulated into a suppository. For suppositories, the vaccine composition

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will include traditional binders and carriers, such as polyalkaline glycols or triglycerides. Such suppositories may be formed from mixtures containing the active ingredient in the range of about 0.5% to about 10% (w/w), preferably about 1% to about 2%.

Protocols for administering to animals the vaccine composition(s) of the present invention are within the skill of the art in view of the present disclosure. Those skilled in the art will select a concentration of the vaccine composition in a dose effective to elicit an antibody and/or T-cell mediated immune response to the antigenic fragment. Within wide limits, the dosage is not believed to be critical. Typically, the vaccine composition is administered in a manner which will deliver between about 1 to about 1,000 micrograms of the subunit antigen in a convenient volume of vehicle, e.g., about 1-10 cc. Preferably, the dosage in a single immunization will deliver from about 1 to about 500 micrograms of subunit antigen, more preferably about 5-10 to about 100-200 micrograms (e.g., 5-200 micrograms).

The timing of administration may also be important. For example, a primary inoculation preferably may be followed by subsequent booster inoculations if needed. It may also be preferred, although optional, to administer a second, booster immunization to the animal several weeks to several months after the initial immunization. To insure sustained high levels of protection against disease, it may be helpful to readminister a booster immunization to the animals at regular intervals, for example once every several years. Alternatively, an initial dose may be administered orally followed by later inoculations, or vice versa. Preferred vaccination protocols can be established through routine vaccination protocol experiments.

The dosage for all routes of administration of *in vivo* recombinant virus vaccine depends on various factors including, the size of patient, nature of infection against which protection is needed, carrier and the like and can readily be determined by those of skill in the art. By way of non-limiting example, a dosage of between 10^3 pfu and 10^8 pfu and the like can be used. As with *in vitro* subunit vaccines, additional dosages can be given as determined by the clinical factors involved.

In one embodiment of the invention, a number of recombinant cell lines are produced according to the present invention by constructing an expression cassette comprising the BAV E1 region and transforming host cells therewith to provide cell lines or cultures expressing the E1 proteins. These recombinant cell lines are capable of allowing a recombinant BAV, having an E1 gene region deletion replaced by heterologous nucleotide sequence encoding for a foreign gene or fragment, to replicate and express the desired foreign gene or fragment thereof which is encoded within the recombinant BAV. These cell lines are also extremely useful in generating recombinant BAV, having an E3 gene deletion replaced by heterologous nucleotide sequence encoding for a foreign gene or fragment, by <u>in vivo</u> recombination following DNA-mediated cotransfection.

In one embodiment of the invention, the recombinant expression cassette can be obtained by cleaving the wild-type BAV genome with an appropriate restriction enzyme to produce a DNA fragment representing the left end or the right end of the genome comprising E1 or E3 gene region sequences, respectively and inserting the left or right end fragment into a cloning vehicle, such as plasmid and thereafter inserting at least one DNA sequence encoding a foreign protein, into E1 or E3 deletion with or without the control of an exogenous

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promoter. The recombinant expression cassette is contacted with the wild-type BAV DNA through homologous recombination or other conventional genetic engineering method within an E1 transformed cell line to obtain the desired recombinant.

The invention also includes an expression system comprising an bovine adenovirus expression vector wherein a heterologous nucleotide, e.g. DNA, replaces part or all of the E3 region and/or part or all of the E1 region. The expression system can be used wherein the foreign nucleotide sequences, e.g. DNA, is with or without the control of any other heterologous promoter.

The BAV E1 gene products of the adenovirus of the invention transactivate most of the cellular genes, and therefore, cell lines which constitutively express E1 proteins can express cellular polypeptides at a higher level than normal cell lines. The recombinant mammalian, particularly bovine, cell lines of the invention can be used to prepare and isolate polypeptides,, including those such as (a) proteins associated with adenovirus E1A proteins: e.g. p300, retinoblastoma(Rb) protein, cyclins, kinases and the like.; (b) proteins associated with adenovirus E1B protein: e.g. p53 and the like.; (c) growth factors, such as epidermal growth factor (EGF), transforming growth factor (TGF) and the like; (d) receptors such as epidermal growth factor receptor (EGF-R), fibroblast growth factor receptor (FGF-R), tumor necrosis factor receptor (TNF-R), insulin-like growth factor receptor (IFG-R), major histocompatibility complex class I receptor and the like; (e) proteins encoded by proto-oncogenes such as protein kinases (tyrosine-specific protein kinases and protein kinases specific for serine or threonine), p21 proteins (guanine nucleotide-binding proteins with GTPase activity and the like; (f) other cellular proteins such as actins, collagens,

fibronectins, integrins, phospholipids, proteoglycans, histones and the like, and (g) proteins involved in regulation of transcription such as TATA-box-binding protein (TBP), TBP-associated factors (TAFs). SP1 binding protein and the like.

The invention also includes a method for providing gene therapy to a mammal in need thereof to control a gene deficiency which comprises administering to said mammal a live recombinant bovine adenovirus containing a foreign nucleotide sequence encoding a non-defective form of said gene under conditions wherein the recombinant virus vector genome is incorporated into said mammalian genome or is maintained independently and extrachromosomally to provide expression of the required gene in the target organ or tissue. These kinds of techniques are recently being used by those of skill in the art to replace a defective gene or portion thereof. Examples of foreign genes nucleotide sequences or portions thereof that can be incorporated for use in a conventional gene therapy include, cystic fibrosis transmembrane conductance regulator gene, human minidystrophin gene, alpha1-antitrypsin gene and the like.

Examples

Described below are examples of the present invention. These examples are provided only for illustrative purposes and are not intended to limit the scope of the present invention in any way. In light of the present disclosure, numerous embodiments within the scope of the claims will be apparent to those of ordinary skill in the art. The contents of the references cited in the specification are incorporated by reference herein.

Cells and viruses

Cell culture media and reagents were obtained from GIBCO/BRL Canada (Burlington, Ontario, Canada). Media were supplemented with 25 mM Hepes and 50 μg/ml gentamicin. MDBK cells or MDBK cells transformed with a plasmid containing BAV3 E1 sequences were grown in MEM supplemented with 10% Fetal bovine serum. The wild-type BAV3 ((strain WBR-1) (Darbyshire et al, 1965 J. Comparative Pathology 75:327) was kindly provided by Dr. B. Darbyshire, University of Guelph, Guelph, Canada) and BAV3-luciferase recombinants working stocks and virus titrations were done in MDBK cells.

Enzymes, bacteria and plasmids

Restriction endonucleases, polymerase chain reaction (PCR) and other enzymes required for DNA manipulations were purchased from Pharmacia LKB Biotechnology (Canada) Ltd. (Dorval, Quebec, Canada), Boehringer-Mannheim, Inc. (Laval or Montreal, Quebec, Canada), New England BioLabs (Beverly, MA), or GIBCO/BRL Canada (Burlington, Ontario, Canada) and used as per manufacturer's instructions. Restriction enzyme fragments of BAV3 DNA were inserted into pUC18 or pUC19 (Yanich-Penon et al (1985) Gene 33:103-109) following standard procedures (Sambrook et al (1989) Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbour Laboratory, New York). *E. coli* strain DH5 (*sup*E44 *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1) was transformed with recombinant plasmids by electroporation (Dower et al. (1988) Nuc. Acids Res., 16:6127-6145). Plasmid DNA was prepared using the alkaline lysis procedure (Bernboim and Doly (1978) Nuc. Acids Res., 7:1513-1523). The plasmid, pSVOA/L containing the entire cDNA encoding firefly luciferase (de Wet et al (1987) Mol. Cell. Biol. 7:725-

737), was a gift from D.R. Helinski, University of California, San Diego, La Jolla, CA.

Construction of recombinant BAV3

MDBK cells transformed with a plasmid containing BAV3 E1 sequences were cotransfected with the wt BAV3 DNA digested with PvuI and the plasmid, pSM51-Luc (Figs. 9 and 10) using the lipofection-mediated cotransfection protocol (GIBCO/BRL, Life Technologies, Inc., Grand Island, NY). The virus plaques produced following cotransfection were isolated, plaque purified and the presence of the luciferase gene in the BAV3 genome was detected by agarose gel electrophoresis of recombinant virus DNA digested with appropriate restriction enzymes.

Southern blot and hybridization

Mock or virus-infected MDBK cells were harvested in lysis buffer (500 μg/ml pronase in 0.01 M Tris, pH 7.4, 0.01 M EDTA, 0.5% SDS) and DNA was extracted (Graham et al (1991) Manipulation of adenovirus vectors In: Methods and Molecular Biology, 7:Gene Transfer and Expression Techniques (Eds. Murray and Walker) Humana Press, Clifton, N.J. pp. 109-128). 100 ng DNA was digested either with BamHI, EcoRI or XbaI and resolved on a 1% agarose gel by electrophoresis. DNA bands from the agarose gel were transferred to a *GeneScreenPlus*™ membrane (Du Pont Canada Inc. (NEN Products), Lachine, Quebec, Canada) by the capillary blot procedure (Southern, E.M. (1975) J. Mol. Biol. 98:503-517). Probes were labeled with ^{32}P using an Oligolabeling Kit (Pharmacia LKB Biotechnology (Canada) Ltd., Dorval, Quebec, Canada) and the unincorporated label was removed by passing the labeled probe through a sephadex G-50 column (Sambrook et al (1989) supra). Probes were kept

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in a boiling water bath for 2 min and used in hybridization experiments following *GeneScreenPlus*™ hybridization protocol. The DNA bands which hybridized with the probe were visualized by autoradiography.

Luciferase assays

The protocol was essentially the same as described (Mittal et al (1993) Virus Res. 28:67-90). Briefly, MDBK cell monolayers in 25 mm multi-well dishes (Corning Glass Works, Corning, NY) were infected in duplicate either with BAV3-Luc (3.1) or BAV3-Luc (3.2) at a m.o.i. of 50 p.f.u. per cell. At indicated time points post-infection, recombinant virus-infected cell monolayers were washed once with PBS (0.137 M NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4) and harvested in 1 ml luciferase extraction buffer (100 mM potassium phosphate, pH 7.8, 1 mM dithiothreitol). The cell pellets were resuspended in 200 µl of luciferase extraction buffer and lysed by three cycles of freezing and thawing. The supernatants were assayed for luciferase activity. For the luciferase assay, 20 µl of undiluted or serially diluted cell extract was mixed with 350 µl of luciferase assay buffer (25 mM glycylglycine, pH 7.8, 15 mM $MgCl_2$, 5 mM ATP) in a 3.5 ml tube (Sarstedt Inc., St-Laurent, Quebec, Canada). Up to 48 tubes can be kept in the luminometer rack and the equipment was programed to inject 100 µl of luciferin solution (1 mM luciferin in 100 mM potassium phosphate buffer, pH 7.8) in the tube present in the luminometer chamber to start the enzyme reaction. The Luminometer (Packard Picolite Luminometer, Packard Instrument Canada, Ltd., Mississauga, Ontario, Canada) used in the present study produced 300 to 450 light units of background count in a 10 sec reaction time. Known amounts of the purified firefly luciferase

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were used in luciferase assays to calculate the amount of active luciferase present in each sample.

Western blotting

Mock or virus-infected MDBK cells were lysed in 1:2 diluted 2X loading buffer (80 mM Tris-HCl, pH 6.8, 0.67 M urea, 25% glycerol, 2.5% SDS, 1 M mercaptoethanol, 0.001% bromophenol blue), boiled for 3 min and then centrifuged to pellet cell debris. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 0.1% SDS-10% polyacrylamide gels (Laemmli, et al (1970) Nature 227:680-685). After the end of the run, polypeptide bands in the gel were electrophoretically transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Richmond, CA). The membrane was incubated at room temperature for 2 h with 1:4000 diluted rabbit anti-luciferase antibody (Mittal et al (1993) supra). The binding of anti-luciferase antibody to the specific protein band/s on the membrane was detected with 1:5000 diluted horseradish peroxidase conjugated-goat anti-rabbit IgG (Bio-Rad Laboratories, Richmond, CA) and with an ECL Western blotting detection system (Amersham Canada Ltd., Oakville, Ontario).

Example 1 Cloning of BAV3 E1 Region DNA for sequencing

To complement the restriction site (Kurokawa et al, 1978 J. Virol., 28:212-218; Hu et al, 1984 J. Virol. 49:604-608) other restriction enzyme sites in the BAV3 genome were defined. The 8.4 kilobase pair (kb) SalI B fragment which extends from the left end of the genome to approximately 24% was cloned into the SmaI-SalI sites of pUC18 essentially as described previously (Graham et al, 1989 EMBO Journal 8:2077-2085). Beginning at the left end of the BAV3 genome, the relevant restriction sites used for subsequent subcloning and their approximate

positions are: SacI (2%), EcoRI (3.5%), HindIII (5%), SacI (5.5%), SmaI (5.6%) and HindIII (11%). Through the use of appropriate restriction enzymes, the original plasmid was collapsed to contain smaller inserts which could be sequenced using the pUC universal primers. Some fragments were also subcloned in both pUC18 and pUC19 to allow confirmational sequencing in both directions. These procedures, together with the use of twelve different oligonucleotide primers hybridizing with BAV3 sequences, allowed to sequence the BAV3 genome from its left end to the HindIII site at 11%.

To ensure that some features of the sequence obtained were not unique to the initial clone selected for sequencing, two more pUC19 clones were prepared containing the SalI fragment from a completely independent DNA preparation. These clones were used to confirm the original sequence for the region from approximately 3% to 5.5% of the BAV3 genome.

DNA sequencing reactions were based on the chain-termination method (Sanger et al. 1977 PNAS, USA 74:5463-5467) and manual sequencing followed the DNA sequencing protocol described in the Sequenase™ kit produced by US Biochemical. [α-^{35}S]dATPs was obtained from Amersham Canada Ltd. All oligonucleotides used as primers were synthesized by the Central Facility of the Molecular Biology and Biotechnology Institute (MOBIX) at McMaster University, Hamilton, Ontario. The entire region (0 to 11%) of the BAV3 genome was sequenced by at least two independent determinations for each position by automated sequencing on a 373A DNA Sequencer (Applied Biosystems) using Taq-Dye terminators. Over half of the region was further sequenced by manual procedures to confirm overlaps and other regions of interest.

DNA sequence analysis and protein comparisons were carried out on a MICROGENIE program.

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Example 2 Coding Sequences of the BAV3 E1 Region

BAV3 genomic DNA, from the left end of the genome to the HindIII site at approximately 11%, was cloned into plasmids and sequenced by a combination of manual and automated sequencing. An examination of the resultant BAV3 E1 genomic sequence (Fig 1) revealed a number of interesting features relevant both to transactivation and to other functions associated with adenovirus E1 proteins. On the basis of open reading frames (ORFs) it was possible to assign potential coding regions analogous to those defined in human Ad5 (HAd5). As shown in Fig 1, ORFs corresponding roughly to the first exon and unique region of HAd5 E1A as well are ORFs corresponding to the 19k and 58k proteins of E1B and the ORF corresponding to protein IX were all defined in this sequence. The open reading frame defining the probable E1A coding region begins at the ATG at nt 606 and continues to a probable splice donor site at position 1215. The first consensus splice acceptor site after this is located after nt 1322 and defines an intron of 107 base pairs with an internal consensus splice branching site at position 1292. The putative BAV3 E1A polypeptide encoded by a message corresponding to these splice sites would have 211 amino acids and a unmodified molecular weight of 23,323. The major homology of the protein encoded by this ORF and HAd5 E1A is in the residues corresponding to CR3 (shown in Fig 2). The homology of amino acid sequences on both sides of the putative intron strengthens the assignment of probable splice donor and acceptor sites. The CR3 has been shown to be of prime importance in the transactivation activity of HAd5 EIA gene products. As seen in Fig. 2A the homology of this sequence in the BAV3 protein to the corresponding region of the 289R E1A protein of HAd5 includes complete conservation of the $CysX_2CysX_{13}CysX_2Cys$

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sequence motif which defines the metal binding site of this protein (Berg, 1986 Science 232:485-487) as well as conservation of a number of amino acids within this region and within the promoter binding region as defined by Lillie and Green 1989 Nature 338:39-44).

The only other region of significant homology between the BAV3 E1A protein and that of HAd5 was a stretch of amino acids known to be important in binding of the cellular Rb protein to the HAd5 E1A protein (Dyson et al, 1990 J. Virol. 64:1353-1356). As shown in Fig 2B, this sequence, which is located between amino acids 120 and 132 in the CR2 region of HAd5 E1A, is found near the amino (N-) terminus of the BAV3 protein between amino acids 26 and 37.

An open reading frame from the ATG at nt 1476 to the termination signal at 1947 defines a protein of 157 amino acids with two regions of major homology to the HAd5 E1B 19k protein. As shown in Fig 3 both the BAV3 and the HAd5 proteins have a centrally located hydrophobic amino acid sequence. The sequence in BAV3, with substitutions of valine for alanine and leucine for valine, should result in a somewhat more hydrophobic pocket than the corresponding HAd5 region. The other portion of HAd5 19k that may be conserved in the BAV3 protein is the serine rich sequence found near the N-terminus (residues 20 to 26) in HAd5 19k and near the C-terminus (residues 136 to 142) in the BAV3 protein (also shown in Fig 3).

On ORF beginning at the ATG at nt 1850 and terminating at nt 3110 overlaps the preceding BAV3 protein reading frame and thus has the same relationship to it as does the HAd5 E1B 56k protein to E1B 19k protein. As shown in Fig 4 this BAV3 protein of 420R and the corresponding HAd5 E1B 56k protein of 496R show considerable sequence homology over their C-terminal 346

residues. The N-terminal regions of these proteins (not depicted in the figure) show no significant homology and differ in overall length.

Following the E1B ORFs, the open reading frame beginning at nt 3200 and ending at the translation terminator TAA at nt 3575 defines a protein of 125R with an unmodified molecular weight of 13,706. As seen in Fig 5 this protein shares some homology with the structural protein IX of HAd5 particularly in N-terminal sequences.

Possible Transcription Control Regions in BAV3 E1

The inverted terminal repeats (ITR) at the ends of the BAV3 genome have been shown to extend to 195 nt (Shinagawa et al, 1987 Gene 55:85-93). The GC-rich 3′ portion of the ITR contains a number of consensus binding sites for the transcription stimulating protein SP1 (Dynan and Tijan (1983) Cell 35:79-87) and possible consensus sites for the adenovirus transcription factor (ATF) (Lee et al. (1987) Nature 325:368-372) occur at nts 60 and 220. While there are no exact consensus sites for the factors EF-1A (Bruder and Healing (1989) Mol. Cell Biol. 9:5143-5153) or E2F (Kovesdi et al, 1987 PNAS, USA 84:2180-2184) upstream of the ATG at nt 606, there are numerous degenerate sequences which may define the enhancer region comparable to that seen in HAd5 (Hearing and Shenk, 1986 Cell 45:229-236).

The proposed BAV3 E1A coding sequence terminates at a TGA residue at nt 1346 which is located within a 35 base pair sequence which is immediately directly repeated (see Fig 1). Two repeats of this sequence were detected in three independently derived clones for a plaque purified stock of BAV3. The number of direct repeats can vary in any BAV3 population though plaque purification allows for isolation of a relatively

homogeneous population of viruses. That direct repeats in the sequences can function as promoter or enhancer elements for E1B transcription is being tested. There are no strong polyA addition consensus sites between the E1A and the E1B coding sequences and in fact no AATAA sequence is found until after the protein IX coding sequences following E1B. The TATAAA sequence beginning at nt 1453 could function as the proximal promoter for E1B but it is located closer to the ATG at 1476 than is considered usual (McKnight et al, 1982 Science 217:316-322). The TATA sequence located further upstream immediately before the proposed E1A intron sequence also seems inappropriately positioned to serve as a transcription box for the E1B proteins. There are clearly some unique features in this region of the BAV3 genome.

The transcriptional control elements for the protein IX transcription unit are conventional and well defined. Almost immediately following the open reading frame for the larger E1B protein there is, at nt 3117, a SP1 binding sequence. This is followed at 3135 by a TATAAAT sequence which could promote a transcript for the protein IX open reading frame beginning at the ATG at 3200 and ending with the TAA at 3575. One polyA addition sequence begins within the translation termination codon and four other AATAA sequences are located at nts 3612, 3664, 3796 and 3932.

In keeping with the general organization of the E1A region of other adenoviruses, the BAV3 E1A region contains an intron sequence with translation termination codons in all three reading frames and which is therefore probably deleted by splicing from all E1A mRNA transcripts. The largest possible protein produced from the BAV3 E1A region will have 211 amino acid residues and is the equivalent of the 289 amino acid protein

translated from the 13s mRNA of HAd5. Two striking features in a comparison of these proteins are the high degree of homology in a region corresponding to CR3 and the absence in BAV3 of most of amino acids corresponding to the second exon of HAd5. In fact the only amino acids encoded in the second exon of BAV3 are, those which are considered to constitute part of CR3. A great deal of work carried out with HAd5 has identified the importance of the CR3 sequences in transactivation of other HAd5 genes. While a detailed analysis of the corresponding BAV3 region and its possible role in transactivation of BAV3 genes needs to be carried out, it is none-the-less interesting to note a couple of possibly pertinent features. The HAd5 CR3 region has been operationally subdivided into three regions (Lillie et al, 1989 Nature 338:39-44; see Fig 8); an N-terminal region from 139 to 153 which has four acidic residues and is thought to be important in transcription activation, a central, metal-binding, region defined by the Cys-X_2-Cys-X_{13}-CysX_2-Cys sequence which is essential for both promoter binding and activation, and a C-terminal region (residues 175-189) which is essential for promoter binding. Since, in most instances, E1A protein is thought not to interact directly with DNA (Ferguson et al 1985), the promoter binding regions may be involved in forming associations with proteins which then allow association with DNA. In Fig 2a the BAV3 E1A protein contains the central, metal binding domain and has considerable homology in the carboxy portion of this region. The BAV3 E1A protein also shows identity of sequence with HAd5 in the carboxy 6 amino acids of the promoter binding domain. These features may allow the BAV3 E1A protein to interact with the same transcription activating factors required for HAd5 E1A function. In contrast, except for a Glu-Glu pair there is little homology between the bovine and

human viruses in the activation domain. The fact that this domain can be functionally substituted by a heterologous acidic activation sequence (Lillie et al, 1989 supra) suggests that protein specificity is not required in this region and this may allow the BAV3 E1A protein to function in the activation of BAV3 genes. The BAV3 E1A activation region contains six acidic residues in the 18 residues amino to the metal binding domain.

The other interesting feature of BAV3 E1A, which is undoubtedly relevant to the oncogenic potential of this virus, is the presence of the sequence Asp27-Leu-Glu-Cys-His-Glu which conforms to, a core sequence known to be important in the binding of cellular Rb and related proteins by the transforming proteins of a number of DNA tumour viruses (Dyson et al, 1990 supra). From deletion mutant analysis there is a clear association between the potential of HAd5 E1A proteins to bind Rb and the ability of the protein to induce morphological transformation in appropriate cells (see references in Dyson et al, 1990 supra). The BAV3 E1A protein is distinct from its HAd5 counterpart in the relative position of this Rb binding sequence which is in the CR2 of HAd5 E1A and near the N-terminus of the BAV3 E1A protein.

Through the use of alternative splice sites HAd5 E1A transcripts can give rise to at least 5 distinct mRNA species (Berk et al, 1978 Cell 14:695-711; Stephens et al, 1987 EMBO Journal 6:2027-2035). Whether BAV3, like HAd5, can generate a number of different mRNA species through the use of alternative splice sites in the E1A transcripts remains to be determined. For example a potential splice donor site which could delete the sequence equivalent to the unique sequence of HAd5 is present immediately after nt 1080 but it is not known if this site is actually used.

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HAd5 E1B encodes two proteins (19k and 56k) either of which can cooperate with E1A, by pathways which are additive and therefore presumably independent (McLorie et al, 1991 J. Gen. Virol. 72:1467-1471), to produce morphological transformation of cells in culture (see for example: Branton et al, 1985 supra; Graham, 1984 supra). The significance of the conservation of the hydrophobic stretch of amino acids in the central portion of the shorter E1B proteins of HAd5 and BAV3 is not clear as yet. A second short region of homology Gln-Ser-Ser-X-Ser-Thr-Ser at residue 136 near the C-terminus of the BAV3 protein is located near the N-terminus at residue 20 in the HAd5 19k protein. The major difference in both length and sequence of the larger (420R) E1B protein of BAV3 from the corresponding HAd5 protein (496R) is confined to the N-terminus of these proteins. The two proteins show considerable evolutionary homology in the 345 amino acids that extend to their C-termini. A similar degree of homology extends into the N-terminal halves of protein IX of BAV3 and HAd5. Taken together these analyses suggest that while BAV3 and the human adenoviruses have diverged by simple point mutational events in some regions, more dramatic genetic events such as deletion and recombination may have been operating in other regions particularly those defining the junction between E1A and E1B.

Example 3 Cloning and sequencing of the BAV3 E3 and fibre genes

The general organization of adenovirus genomes seems to be relatively well conserved so it was possible to predict, from the locations of a number of HAd E3 regions, that BAV E3 should lie between map units (m.u.) 77 to 86. To prepare DNA for cloning and sequencing, BAV3 (strain WBR-1) was grown in Madin-Darby bovine

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kidney (MDBK) cells, virions were purified and DNA was extracted (Graham, F.L. & Prevec, L. (1991) Methods in Molecular Biology, vol. 7, Gene Transfer and Expression Protocols, pp. 109-146. Edited by E.J. Murray, Clifton, New Jersey; Humana Press.). Previously published restriction maps for *Eco*RI and *Bam*HI (Kurokawa et al., 1978) were confirmed (Fig. 6). The *Bam*HI D and *Eco*RI F fragments of BAV3 DNA were isolated and inserted into pUC18 and pUC19 vectors, and nested sets of deletions were made using exonuclease III and S1 nuclease (Henikoff, S. (1984) Gene, 28:351-359). The resulting clones were sequenced by the dideoxynucleotide chain termination technique (Sanger, F., Nicklen, S. & Coulson, A.R. (1977) Proceedings of the National Academy of Sciences, U.S.A., 74:5463-5467). The nucleotide sequence from positions 1 to 287 was obtained from the right end of the *Bam*HI B fragment (Fig. 6). The sequence of the regions spanning (i) the *Bam*HI site at nucleotide 3306 and the *Eco*RI site at nucleotide 3406, and (ii) the *Eco*RI site at nucleotide 4801 and the nucleotide 5100 was obtained from a plasmid containing the *Xba*I C fragment (m.u. 83 to 100; not shown) using primers hybriding to BAV3 sequences. Analysis of the sequence was performed with the aid of the PC/GENE sequence analysis package developed by Amos Bairoch, Department of Medical Biochemistry, University of Geneva, Switzerland.

The 5100 nucleotide sequence which extends between 77 and 92 m.u. of the BAV3 genome is shown in Fig. 7. The upper strand contains 14 open reading frames (ORFs) which could encode polypeptides of 60 amino acid residues or more (Fig. 6 and 7). The lower strand contains no ORF encoding a protein of longer than 50 amino acids after an initiation codon. The predicted amino acid sequence for each ORF on the upper strand was analyzed for homology with predicted amino acid sequences

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from several sequenced Ads: HAd2 (Hérissé, J., Courtois, G. & Galibert, F. (1980) Nucleic Acids Research, 8:2173-2192; Hérissé, J., Courtois, G. & Galibert, F. (1981) Nucleic Acids Research, 9:1229-1249), -3(Signas, C., Akusjarvi, G. & Pettersson, U. (1985) Journal of Virology, 53:672-678.), -5(Cladaras, C. & Wold, W.S.M. (1985) Virology, 140:28-43), -7 (Hong, J.S., Mullis, K.G. & Engler, J.A. (1988) Virology, 167:545-553) and -35(Flomenberg, P.R., Chen, M. & Horwitz, M.S. (1988) Journal of Virology, 62:4431-4437), and murine Ad1 (MAd1) (Raviprakash, K.S., Grunhaus, A., El Kholy, M.A. & Horwitz, M.S. (1989) Journal of Virology, 63:5455-5458) and canine Ad1 (CAd1) (Dragulev, B.P., Sira, S., Abouhaidar, M.G. & Campbell, J.B. (1991) Virology, 183:298-305). Three of the BAV3 ORFs exhibited homology with characterized HAd proteins pVIII, fibre and the 14.7K E3 protein. The amino acid sequence predicted from BAV3 ORF 1 shows overall identity of approximately 55% when compared to the C-terminal 75% of HAd2 pVIII (Cladaras & Wold, 1985, supra) (Fig. 8a), indicating that ORF 1 encodes the right end of BAd3 pVIII. Near the C-terminal end of BAd3 pVIII there is a 67 amino acid stretch (residues 59 to 125; Fig. 8a) which has 75% identity with HAd2 pVIII. This region has previously been shown to be highly conserved among different Ads (Cladaras & Wold, 1985, supra; Signas, C., Akusjarvi, G. & Pettersson, U. (1986) Gene, 50:173-184,; Raviprakash et al., 1989, supra; Dragulev et al., 1991, supra).

The fibre protein is present on the surface of the virion as long projections from each vertex of the icosahedral capsid and is involved in a number of Ad functions including attachment of the virus to the cell surface during infection, assembly of virions and antigenicity (Philipson, L. (1983) Current Topics in Microbiology and Immunology, 109:1-52). On the basis of

the primary structure of HAd2 fibre protein, it has been proposed that the shaft region (between amino acid residues 40 and 400) is composed of a number of repeating structural motifs containing about 15 hydrophobic residues organized in two short β-sheets and two β-bends (Green, N.M., Wrigley, N.G., Russell, W.C., Martin, S.R. & McLachlan, A.D. (1983) EMBO Journal, 2:1357-1365). The amino acid sequences at the N terminus of the BAV3 ORF 6-encoded protein share about 60% identity with the HAd2 fibre protein tail, but there is little or no similarity in the knob region, and about 45% identity overall (Fig. 8c). The BAd3 fibre gene would encode a protein of 976 residues if no splicing occurs, i.e. 394 amino acid residues longer than the HAd2 fibre protein. The number of repeating motifs in the shaft region of the fibre protein from different Ads varies between 28 and 23 (Signas et al., 1985, supra; Chroboczek, J. & Jacrot, B. (1987) Virology, 161:549-554; Hong et al., 1988, supra; Raviprakash et al., 1989, supra; Dragulev et al., 1991, supra). The BAV3 fibre protein can be organized into 52 such repeats in this region (not shown), which would account for most of the difference in size compared to those of HAd2, HAd3, HAD5, HAd7, CAd1 and MAd1 (Signas et al., 1985,supra; Hérissé et al., 1980,supra; Hérissé & Galibert, 1981, supra; Hong et al., 1988,supra; Raviprakash et al., 1989, supra; Dragulev et al., 1991, supra).

HAd2 and HAd5 E3 lies between the pVIII and the fibre genes an encodes at least 10 polypeptides (Cladaras & Wold, 1985,supra). The promoter for E3 of these two serotypes lies within the sequences encoding pVIII, about 320 bp 5′ of the termination codon. No consensus TATA box is found in the corresponding region of the BAV3 sequences. A non-canonical polyadenylation signal (ATAAA) for E3 transcripts is located at position 1723,

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between the end of the putative E3 region and the beginning of ORF 6, encoding the fibre protein, and two consensus signals are located within ORF 6 at positions 2575 and 3565. The polyadenylation signal for the fibre protein is located at nucleotide 4877. Six ORFs were identified in the BAV3 genome between the pVIII and the fibre genes, but only four (ORFs 2, 3, 4 and 5) have the potential to encode polypeptides of at least 50 amino acids after an initiation codon (Fig. 7). The amino acid sequence predicted to be encoded by ORF 2 is 307 residues long and contains eight potential N-glycosylation sites (Fig. 7) as well as a hydrophobic sequence which may be a potential transmembrane domain (PLLFAFVLCTGCAVLLTAFGPSILSGT) between residues 262 and 289. This domain may be a part of the protein homologous to the HAd2 and HAd5 19K E3 glycoprotein (Cladaras & Wold, 1985, supra), and the proposed CAd1 22.2K protein (Dragulev et al., 1991, supra), but ORF 2 does not show appreciable homology with these proteins. The ORF 4 shows approximately 44% identity with the 14.7K E3 protein of HAd5 (Fig. 6 and 8b), which has been shown to prevent lysis of virus-infected mouse cells by tumour necrosis factor (Gooding, L.R., Elmore, L.W., Tollefson, A.E., Brody, H.A. & Wold, W.S.M. (1988) Cell, 53:341-346; Wold, W.S.M. & Gooding, L.R. (1989) Molecular Biology and Medicine, 6:433-452). Analysis of the 14.7K protein sequence from HAd2, -3, -5 and -7 has revealed a highly conserved domain, which in HAd5 lies between amino acid residues 41 and 56 (Horton, T.M., Tollefson, A.E., Wold, W.S.M. & Gooding, L.R. (1990) Journal of Virology, 64:1250-1255). The corresponding region in the BAV3 ORF 4-encoded protein, between amino acids 70 and 85, contains 11 amino acids identical to those of the HAd5 14.7K protein conserved domain (Fig. 8b).

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The BAV3 E3 region appears to be approximately 1.5kbp long, about half the size of those of HAd2 and -5 (Cladaras & Wold, 1985, supra), and novel splicing events in BAV3 E3 would be required to generate more homologues to the HAd3 E3 proteins. A similarly short E3 region has been reported for MAd1 (RAviprakash et al., 1989, supra) and CAd1 (Dragulev et al., 1991,supra).

Example 4 Construction of BAV3-luciferase recombinants

Adenovirus-based mammalian cell expression vectors have gained tremendous importance in the last few years as a vehicle for recombinant vaccine delivery, and also in gene therapy. BAV3-based expression vectors have a greater potential for developing novel recombinant vaccines for veterinary use. To show that BAV3 E3 gene products are not essential for virus growth in cultured cells and this locus could be used to insert foreign DNA sequences, a 1.7 kb fragment containing the firefly luciferase gene was introduced in the 696 bp deletion of the E3 region of the BAV3 genome in the E3 parallel orientation to generate a BAV3 recombinant.

The rationale of using the luciferase gene is that it acted as a highly sensitive reporter gene when introduced in the E3 region of the HAd5 genome to generate HAd5-Luc recombinants (Mittal et al (1993) Virus Res. 28:67-90).

To facilitate the insertion of the firefly luciferase gene into the E3 region of the BAV3 genome, a BAV3 E3 transfer vector containing the luciferase gene was constructed (Fig. 9). The BAV3 E3 region falls approximately between m.u. 77 and 82. In our first series of vectors we replaced a 696 bp XhoI-NcoI E3 deletion (between m.u. 78.8 and 80.8) with a NruI-SalI cloning sites for insertion of foreign genes to obtain pSM14del2. A 1716 bp BsmI-SspI fragment

containing the luciferase gene was isolated and first inserted into an intermediate plasmid, pSM41, in the E3 locus at the SalI site by blunt end ligation to generate pSM41-Luc. The luciferase gene without any exogenous regulatory sequences, was inserted into the E3 locus in the same orientation as the E3 transcription unit. The kan^r gene was inserted into pSM41-Luc at the XbaI site present within the luciferase gene to generate an amp^r/kan^r plasmid, pSM41-Luc-Kan. A 7.7 kb fragment containing the BAV3 sequences along with the luciferase gene and the kan^r gene was obtained from pSM41-Luc-Kan by digestion with BamHI and inserted into an amp^r plasmid, pSM51 partially digested with BamHI to replace a 3.0 kb BamHI fragment (lies between m.u. 77.8 and 86.4) to generate a doubly resistant (kan^r & amp^r) plasmid, pSM51-Luc-Kan. The kan^r gene was deleted from pSM51-Luc-Kan by partial cleavage with XbaI to generate pSM51-Luc containing the luciferase gene in the E3-parallel orientation.

MDBK cells transformed with a plasmid containing the BAV3 E1 sequences was cotransfected with the wt BAV3 DNA digested with PvuI, which make two cuts within the BAV3 genome at m.u 65.7 and 71.1, and the plasmid, pSM51-Luc to rescue the luciferase gene in E3 of the BAV3 genome by *in vivo* recombination (Fig. 10). The digestion of the wt BAV3 DNA with PvuI was helpful in minimizing the generation of the wt virus plaques following cotransfection. The left end of the wt BAV3 genome represented by PvuI 'A' fragment falls between m.u. 0 and 65.7, and pSM51-Luc which extends between m.u. 31.5 and 100 (except for E3 deletion replaced with the luciferase gene) have sufficient overlapping BAV3 DNA sequences to generate recombinant viruses.

Two virus plaques were obtained in two independent cotransfection experiments which were grown

in MDBK cells. The viral DNA from both plaques was extracted and analyzed by agarose gel electrophoresis after digesting either with BamHI, EcoRI or XbaI to identify the presence and orientation of the luciferase gene in the viral genome (data not shown). In the genomes of both recombinants, the luciferase gene was present in the E3 region in the E3 parallel orientation. The BAV3-luciferase recombinants were plaque purified and named BAV3-Luc (3.1) and BAV3-Luc (3.2) to represent plaques obtained from two independent experiments. Since both recombinant virus isolates were identical they will be referred to as BAV3-Luc. The presence of the luciferase gene in BAV3-Luc isolates are further confirmed by Southern blot analyses and luciferase assays using extracts from recombinant virus-infected cells.

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Characterization of BAV3-recombinants

Southern blot analyses of the wt BAV3 and recombinants genomic DNA digested either with BamHI, EcoRI or XbaI, were carried out to confirm the presence and orientation of the luciferase gene in the E3 locus and the deletion of the 696 bp XhoI-NcoI fragment from E3 of the BAV3-Luc genome (Fig. 11). When the blot was probed with a 696 XhoI-NcoI fragment of E3 of the BAV3 genome (panel A, lanes 4 to 9) no hybridization signal was detected with the DNA fragments from the recombinant viruses, however, the expected bands (3.0 kb BamHI, 8.1 kb EcoRI, and 18.5 kb XbaI) of the wt BAV3 DNA fragments (panel A, lanes 10 to 12) showed hybridization, confirming that the 696 bp XhoI-NcoI fragment of the E3 region was indeed deleted in the BAV3-Luc genomic DNA. In panel B, when an identical blot was probed with the luciferase gene, there were strong hybridization signals with the DNA fragments from the recombinant viruses (4.0 kb BamHI (lane 4 & 7), 6.0 kb & 3.2 kb EcoRI (lanes 5 &

8), 16.7 kb & 2.9 kb XbaI (lanes 6 & 9)). These results confirmed that the BAV3-Luc contains the luciferase gene in the E3 parallel orientation with a 696 bp XhoI-NcoI E3 deletion.

The growth characteristics of the recombinant viruses was compared with the wt BAV3 in a single step growth curve (Fig. 12). Virus titers in MDBK cells-infected with the wt BAV3 started increasing at 12 h post-infection reaching a maximum at 36-48 h post-infection and then declined thereafter. Virus titers of the recombinant viruses also started increasing at 12 h postinfection reaching a maximum at 48 h post-infection and then declined, however, the titers of recombinant viruses remained approximately one log lower than the wt virus. The plaque size of the recombinant viruses were also comparatively smaller than the wt virus (data not shown).

Kinetics of luciferase expression by BAV3-Luc

Luciferase activity in BAV3-Luc-infected MDBK cells was monitored at different times post-infection by luciferase assays (Fig. 13). A low level of luciferase activity was first observed at 12 h post-infection reaching a peak at 30 h post-infection and then dropped subsequently. At 30 h post-infection, approximately 425 pg luciferase was detected in $4x10^5$ BAV3-Luc (3.1)--infected MDBK cells. In MDBK cells-infected with the wt BAV3, luciferase expression was not detected (data not shown). The kinetics of luciferase expression by BAV3-Luc (3.1) and BAV3-Luc (3.2) appears very much similar. The kinetics of luciferase expression also showed that the majority of enzyme expression in virus-infected cells seemed to occur late in infection. To determine luciferase expression in the absence of viral DNA replication, BAV3-Luc-infected MDBK cells were incubated

in the presence of an inhibitor of DNA synthesis, 1-β-D--arabinofuranosyl cytosine (AraC) and luciferase activity was measured in virus-infected cell extracts at various times post-infection and compared to luciferase expression obtained in the absence of AraC (Fig. 14). When the recombinant virus-infected cells were incubated in the presence of AraC, luciferase expression at 18, 24 and 30 h post-infection was approximately 20-30% of the value obtained in the absence of AraC. These results indicated that the majority of luciferase expression in MDBK cells infected with BAV3-Luc took place after the onset of viral DNA synthesis. To confirm this MDBK cells-infected with the BAV3-Luc were grown in the absence or presence of AraC, harvested at 18 h, 24 h, and 30 h post-infection, viral DNA extracted and analyzed by dot bot analysis using pSM51-Luc (see Fig. 9) as a probe (data not shown). In the presence of AraC, viral DNA synthesis was severely reduced compared to viral DNA synthesis in the absence of AraC.

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Western blot analysis of BAV3-Luc-infected cells

Luciferase was expressed as an active enzyme as determined by luciferase assays using extracts from MDBK cells-infected with BAV3-Luc (see Fig. 13). The luciferase gene without any exogenous regulatory sequences was inserted into E3 of the BAV3 genome, therefore, there was a possibility of luciferase expression as a fusion protein with part of an E3 protein if the luciferase gene was in the same frame, Such as, F1 and F3 which represent open reading frames (ORFs) for E3 proteins (Fig. 15) or the fusion protein may arise due to recognition of an upstream initiation codon in the luciferase ORF. To explore this possibility we sequenced the DNA at the junction of the luciferase gene and the BAV3 sequences with the help of a plasmid, pSM51-Luc and

a synthetic primer design to bind luciferase coding sequences near the initiation codon (data not shown). The luciferase coding region fell in frame F2. The luciferase initiation codon was the first start codon in this frame, however, the ORF started at 84 nucleotides upstream of the luciferase start codon. To further confirm that luciferase protein is of the same molecular weight as purified firefly luciferase, unlabeled mock-infected, wt BAV3-infected or BAV3-Luc-infected MDBK cell extracts were reacted with an anti-luciferase antibody in a Western blot (Fig. 16). A 62 kDa polypeptide band was visible in the BAV3-Luc (lane 3 and 4)-infected cell extracts which were of the same molecular weight as pure firefly luciferase (lane 5). We are not sure whether a band of approximately 30 kDa which also reacted with the anti-luciferase antibody in lanes 3 and 4 represented a degraded luciferase protein.

The majority of luciferase expression is probably driven from the major late promoter (MLP) to provide expression paralleling viral late gene expression, moreover, the enzyme expression seen in the presence of AraC may be taking place from the E3 promoter. In HAd5 vectors, foreign genes without any exogenous regulatory sequences when inserted in E3 also displayed late kinetics and were inhibited by AraC. The BAV3 recombinant virus replicated relatively well in cultured cells but not as good as the wt BAV3. This is not surprising as infectious virus titers of a number of HAd5 recombinants were slightly lower than the wt HAd5 (Bett et al (1993) J. Virol. 67:5911-5921). This may be because of reduced expression of fiber protein in recombinant adenoviruses having inserts in the E3 region compared to the wt virus (Bett et al, supra and Mittal et al (1993) Virus Res. 28:67-90).

The E3 of BAV3 is approximately half the size of the E3 region of HAd2 or HAd5 and thus has the coding potential for only half the number of proteins compared to E3 of HAd2 or HAd5 (Cladaras et al (1985) Virology 140:28-43: Herisse et al (1980) Nuc. Acids Res. 8:2173-2192; Herisse et al (1981) Nuc. Acids Res. 9:1229-1249 and Mittal et al (1993 J. Gen. Virol. 73:3295-3000). BAV3 E3 gene products have been shown to be not required for virus growth in tissue culture. However, presently it is known that BAV3 E3 gene products also evade immune surveillance *in vivo* like HAds E3 proteins. One of the BAV3 E3 open reading frames (ORFs) has been shown to have amino acid homology with the 14.7 kDa E3 protein of HAds (Mittal et al (1993) supra). The 14.7 kDa E3 protein of HAds prevents lysis of virus-infected mouse cells by tumour necrosis factor (Gooding et al (1988) Cell 53:341-346 and Horton et al (1990) J. Virol. 64:1250-1255). The study of pathogenesis and immune responses of a series of BAV3 E3 deletion mutants in cattle provides very useful information regarding the role of E3 gene products in modulating immune responses in their natural host.

The BAV3-based vector has a 0.7 kb E3 deletion which can hold an insert up to 2.5 kb in size. The BAV3 E3 deletion can extend probably up to 1.4 kb which in turn would also increase the insertion capacity of this system. The role of the MLP and the E3 promoter is examined to determine their ability to drive expression of a foreign gene inserted into E3 when a proper polyadenylation signal is provided. Exogenous promoters, such as, the simian virus 40 (SV40) promoter (Subramant et al (1983) Anal. Biochem. 135:1-15), the human cytomegalovirus immediate early promoter (Boshart et al (1985) Cell 43:215-222), and the human beta-actin promoter (Gunning et al (1987) PNAS, USA 84:4831-4835) are tested to evaluate their ability to facilitate

expression of foreign genes when introduced into E3 of the BAV3 genome.

Recently HAd-based expression vectors are under close scrutiny for their potential use in human gene therapy (Ragot et al (1993) Nature 361:647-650; Rosenfeld et al (1991) Science 252:431-434; Rosenfeld et al (1992) Cell 68:141-155 and Stratford-Perricaudet et al (1990) Hum. Gene. Ther. 1:241-256). A preferable adenovirus vector for gene therapy would be one which maintains expression of the required gene for indefinite or for a long period in the target organ or tissue. It may be obtained if the recombinant virus vector genome is incorporate into the host genome or maintained its independent existence extrachromosomally without active virus replication. HAds replicate very well in human, being their natural host. HAds can be made defective in replication by deleting the E1 region, however, how such vectors would maintain the expression of the target gene in a required fashion is not very clear. Moreover, the presence of anti-HAds antibodies in almost every human being may create some problems with the HAd-based delivery system. The adenovirus genomes have a tendency to form circles in non-permissive cells. BAV-based vectors could provide a possible alternative to HAd-based vectors for human gene therapy. As BAV3 does not replicate in human, the recombinant BAV3 genomes may be maintained as independent circles in human cells providing expression of the essential protein for a long period of time.

The foreign gene insertion in animal adenoviruses is much more difficult than HAds because it is hard to develop a cell line which is also good for adenovirus DNA-mediated transfection. This may be one of the major reasons that the development of an animal adenovirus-based expression system has not been reported

so far. It took us more than a year to isolate a cell line suitable for BAV3 DNA-mediated transfection. However, the rapid implementation of BAV-based expression vectors for the production of live virus recombinant vaccines for farm animals, is very promising. BAVs grow in the respiratory and gastrointestinal tracts of cattle, therefore, recombinant BAV-based vaccines have use to provide a protective mucosal immune response, in addition to humoral and cellular immune responses, against pathogens where mucosal immunity plays a major role in protection.

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Example 5 Generation of cell lines transformed with the BAV3 E1 sequences

MDBK cells in monolayer cultures were transfected with pSM71-neo, pSM61-kan1 or pSM61-kan2 by a lipofection-mediated transfection technique (GIBCO/BRL, Life Technologies, Inc., Grand Island, NY). At 48 h after transfection, cells were maintained in the MEM supplemented with 5% fetal bovine serum and 700 μg/ml G418. The medium was changed every 3rd day. In the presence of G418, only those cells would grow which have stably incorporated the plasmid DNA used in transfection experiments into their genomes and are expressing the neo^r gene. The cells which have incorporated the neo^r gene might also have taken up the BAV3 E1 sequences and thus expressing BAV3 E1 protein/s. A number of neo^r (i.e., G418-resistant) colonies were isolated, expended and tested for the presence of BAV3 E1 message/s by Northern blot analyses using a DNA probe containing only the BAV3 E1 sequences. Expression of BAV3 E1 protein/s were confirmed by a complimentation assay using a HAd5 deletion mutant defective in E1 function due to an E1 deletion.

Fetal bovine kidney cells in monolayers were also transfected with pSM71-neo, pSM61kan-1 or pSM61-kan2 by the lipofection-mediated transfection technique, electroporation (Chu et al (1987) Nucl. Acids Res. 15:1311-1326), or calcium phosphate precipitation technique (Graham et al (1973) Virology 52:456-467). Similarly, a number of G418-resistant colonies were isolated, expended and tested for the presence of BAV3 E1 gene products as mentioned above.

Example 6 Generation of a BAV3 recombinant containing the beta-galactosidase gene as an E1 insert

As E1 gene products are essential for virus replication, adenovirus recombinants containing E1 inserts will grow only in a cell line which is transformed with the adenovirus E1 sequences and expresses E1. A number of cell line which are transformed with the BAV3 E1 sequences were isolated as described earlier. The technique of foreign gene insertions into the E1 regions is similar to the gene insertion into the E3 region of the BAV3 genome, however, for insertion into E1 there is a need of an E1 transfer plasmid which contains DNA sequences from the left end of the BAV3 genome, an appropriate deletion and a cloning site for the insertion of foreign DNA sequences. G418-resistant MDBK cell monolayers were cotransfected with the wild-type (wt) BAV3 DNA and pSM71-Z following the lipofection-mediated transfection procedure (GIBCO/BRL, Life Technologies, Inc., Grand Island, NY). The monolayers were incubated at 37°C under an agarose overlay. After a week post-incubation an another layer of overlay containing 300 ug/ml Blu-gal™ (GIBCO/BRL Canada, Burlington, Ontario, Canada) was put onto each monolayer. The blue plaques were isolated, plaque purified and the presence of the beta-galactosidase gene

in the BAV3 genome was identified by agarose gel electrophoresis of recombinant virus DNA digested with suitable restriction enzymes and confirmed by beta-galactosidase assays using extracts from recombinant virus infected cells.

Deposit of Biological Materials

The following materials were deposited and are maintained with the Veterinary Infectious Disease Organization (VIDO), Saskatoon, Saskatchewan, Canada.

The nucleotide sequences of the deposited materials are incorporated by reference herein, as well as the sequences of the polypeptides encoded thereby. In the event of any discrepancy between a sequence expressly disclosed herein and a deposited sequence, the deposited sequence is controlling.

Material	Internal Accession No.	Deposit Date
Recombinant plasmids		
pSM51	pSM51	Dec 6, 1993
pSM71	pSM71	Dec 6, 1993
Recombinant cell lines		
MDBK cells transformed with BAV3 E1 sequences(MDBK-BAVE1)		Dec 6, 1993
Fetal bovine kidney cells transformed with BAV3 E1 sequences(FBK-BAV-E1)		Dec 6, 1993

While the present invention has been illustrated above by certain specific embodiments, the specific examples are not intended to limit the scope of the invention as described in the appended claims.